

Colchicine analogues that bind reversibly to tubulin define microtubular requirements for newly synthesized protein secretion in rat lacrimal gland

(microtubule network)

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ABSTRACT The role of microtubules in ³H-labeled protein secretion in rat lacrimal glands was probed by the use of colchicine and two of its analogues that reversibly bind to tubulin. These analogues were 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatriene-1-one and 2,3,4,4'-tetramethoxy-1,1'-biphenyl, the latter having been synthesized for these studies. Immunofluorescence revealed that untreated exocrine acinar cells contained an intact microtubule network, which was totally abolished by drug addition. Subsequent drug removal restored the network for the two reversibly binding drugs—more rapidly so for the biphenyl, but this was not the case with colchicine. The protein-secretory process was examined by adding the three drugs at various stages—prepulse incubation, pulse, maturation, apical storage of granules, and discharge under cholinergic stimulation. Comparison with the kinetics of microtubular network restoration, which differed for the two reversibly binding drugs, led to the conclusion that the microtubular system is critical to the maturation phase of secretion.

The practical irreversibility of colchicine binding to tubulin (1) limits considerably the use of this drug as a probe of cellular mechanisms because its effects are essentially all-or-none in character. Recently, several analogues of colchicine have been synthesized (refs. 2 and 4; M.J.G., unpublished results) that bind reversibly to pure tubulin in the colchicine-binding site and inhibit microtubule assembly reversibly at substoichiometric levels (3, §). It appeared, therefore, that these colchicine analogues should be much more sensitive probes of the linkage between protein secretory-process stages and microtubular integrity than the parent drug. In previous studies (5), we proposed that perturbation of the microtubular network by colchicine induces a slow-down of secretory-protein transport from ribosomal endoplasmic reticulum to Golgi region and inhibition of the discharge of secretory granules formed under these conditions. Therefore, microtubules could interfere with the steps of formation, maturation, and apical storage of new secretory granules. From our earlier hypothesis, addition of the reversibly bound colchicine analogues would be predicted to disrupt the cellular microtubule network and consequently arrest the secretory process. Removal of these drugs should reverse such effects if inhibition is caused solely by the absence of microtubules.

To test this hypothesis parallel studies were undertaken on the effects of such colchicine analogues on (i) integrity of the microtubule network in acini and (ii) exocrine secretion of protein in lobules from rat exorbital lacrimal glands. The

results obtained with two drugs that are progressively simplified analogues of colchicine are reported in this paper. The drugs are 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatriene-1-one (MTC) (2) (structure II) and 2,3,4,4'-tetramethoxy-1,1'-biphenyl (TMB) (M.J.G., unpublished results) (structure III). MTC is the colchicine molecule from which ring B had been removed, and TMB is MTC from which the carboxyl of ring C had been excised. *In vitro*, both inhibit microtubule formation reversibly—MTC at a concentration level of 1 μ M (3) and TMB at concentrations ten times higher (§).

MATERIALS AND METHODS

Colchicine was obtained from Calbiochem, carbachol was obtained from Mann Research Laboratories (New York), and collagenase (CLS III) was obtained from Worthington. MTC was a gift from T. J. Fitzgerald (Florida A & M University, Tallahassee, FL); TMB was synthesized by a procedure described elsewhere (M.J.G., unpublished work).

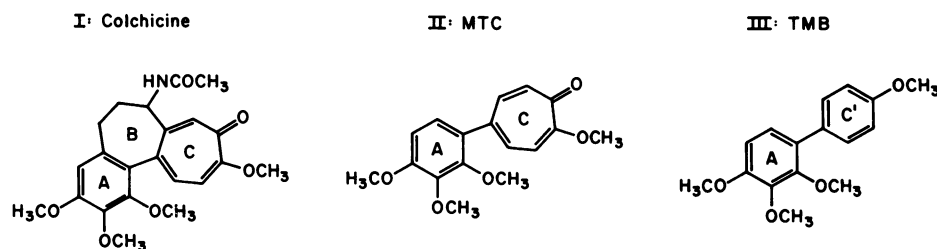
Male Sprague-Dawley rats (5–6 weeks old) were used throughout this study. For the immunofluorescence and secretion experiments, the lacrimal exorbital glands were rapidly dissected and fragmented.

Immunofluorescence. Acini were prepared after digestion with collagenase. Briefly, four glands from two rats were incubated for 45 min in 5 ml of Krebs-Ringer bicarbonate (KRB) buffer/1 mM calcium/0.1% bovine serum albumin (BSA) in the presence of collagenase at 200 units per ml. The acini were dissociated by gentle pipetting (10 times) with a plastic pipette at 15-min intervals. The acini were then filtered through 120- μ m nylon mesh and collected by centrifugation at 50 \times g for 5 min. The acini were then washed further by centrifugation through KRB buffer/4% BSA and finally suspended in 12 ml of KRB buffer. Two milliliters of this acinar preparation was incubated for 70 min in 4 ml of the same medium as used for isolation either with or without drug (1 μ M colchicine, 25 μ M MTC, or 100 μ M TMB). The acini were then washed three times with the same buffer either with or without colchicine and its analogues and incubated for various times up to 145 min under similar conditions. Incubations were stopped by washing in phosphate-buffered saline at room temperature with or without the drugs.

Abbreviations: MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatriene-1-one; TMB, 2,3,4,4'-tetramethoxy-1,1'-biphenyl; KRB, Krebs-Ringer bicarbonate buffer; BSA, bovine serum albumin.

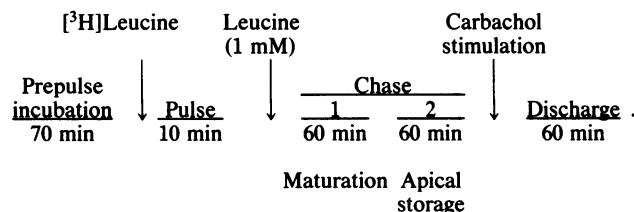
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After incubation, the acini were centrifuged in Cytospin (Shandon Elliott) onto gelatin-dipped microscope slides, fixed, and stained according to a modified procedure of Ash *et al.* (6). Shortly after fixation with 3% (vol/vol) paraformaldehyde/phosphate-buffered saline, the cell membrane was permeabilized with 0.1–0.2% Triton-X 100. The first antibody was an anti- α -tubulin monoclonal antibody (Amersham), diluted 1:1000; the second antibody was a fluorescein-labeled goat anti-mouse IgG (Diagnostics Pasteur), diluted 1:100. The stained acini were examined in an epi-illumination Leitz-dialux photomicroscope equipped with a planapo $\times 63$ oil-immersion objective and the appropriate filter for fluorescein.

^3H -Labeled Protein Secretion. Gland lobules were incubated for 70 min at 37°C in KRB buffer. The lobules were then pulse-labeled with [^3H]leucine for 10 min and incubated for two 60-min periods that overlap the steps of secretory-granule maturation and apical storage, as described (5). The sequence of procedures is summarized by scheme I.



Scheme I

Colchicine ($1\ \mu\text{M}$), MTC ($25\ \mu\text{M}$), or TMB ($100\ \mu\text{M}$) were introduced at various times with respect to the radioactive-tracer addition, as indicated in Table 1. These drugs had no effect on the incorporation of [^3H]leucine into proteins. Between these successive incubations, the gland lobules were thoroughly washed three times with KRB buffer, both with and without drugs. Discharge of labeled proteins occurred for 60 min with or without colchicine or its analogues in both the absence and presence of $2\ \mu\text{M}$ carbachol as stimulant, as described (7). Secretion was expressed as the amount of ^3H -labeled protein present in the incubation medium relative to total ^3H -labeled protein contained in the incubation medium plus the tissue.

RESULTS

Concentrations of the colchicine added to the incubation medium both in the immunofluorescence and in the protein-discharge inhibition experiments were established as described (8). Concentrations of MTC and TMB were established from their potency as inhibitors of *in vitro* microtubule formation from pure tubulin (3, 4, 9) and were $1\ \mu\text{M}$ for colchicine, $25\ \mu\text{M}$ for MTC, and $100\ \mu\text{M}$ for TMB.

Effect on the Microtubule Network. Without drugs the immunofluorescence experiments revealed unambiguously the intact microtubule network in exocrine cells still associated in acini—i.e., in cells that had kept their polarity (Fig. 1). The immunofluorescence pattern of a typical acinus after 220-min incubation at 37°C in KRB/BSA buffer is shown. The microtubule network clearly spans the entire cell, except, of course, for the nucleus.

Effects of 70-min incubation with the drugs are shown in Fig. 2a for colchicine, Fig. 2c for MTC, and Fig. 2e for TMB. Addition of each of the three drugs leads to the total disappearance of the microtubule network. The network fluorescence has been replaced by a weak diffuse fluorescence, which is homogeneously distributed over the entire cytoplasm. Extension of the incubation time with any of the three drugs to 220 min did not alter this pattern (data not shown).

If, after 70-min incubation of the acini with these drugs, the acini were reincubated in a drug-free medium, results differed sharply between colchicine and its two analogues. For colchicine, 145 min of washing had no effect on the immunofluorescence picture; the pattern remained diffuse, as shown in Fig. 2b, resembling that of Fig. 2a. Disruption of the microtubule network by colchicine obviously was not reversed. On the other hand, when the cells that had been incubated for 70 min with either MTC or TMB were washed with drug-free medium, the microtubule network reappeared fully. Kinetics studies have shown that the reappearance of the microtubule network on incubation in a drug-free medium occurred in as little as 25 min for TMB (Fig. 2f) and 40 min for MTC (Fig. 2d). It is evident, therefore, that the two drugs, which *in vitro* inhibit microtubule formation reversibly, also disrupt microtubules within cells reversibly.

Effect on ^3H -Labeled Protein Secretion. Table 1 presents typical results. Timing of drug addition was chosen from previously reported kinetics of intracellular protein transport in lacrimal gland lobules (5). Experimental protocols as outlined in the table yielded the following results:

Experiment A: Colchicine, MTC, or TMB were present during all secretory-process stages—prepulse incubation, pulse, migration and apical storage of secretory granules (chases), and discharge of these granules under cholinergic stimulation. Discharge of labeled proteins was inhibited by $\approx 70\%$ for all three drugs.

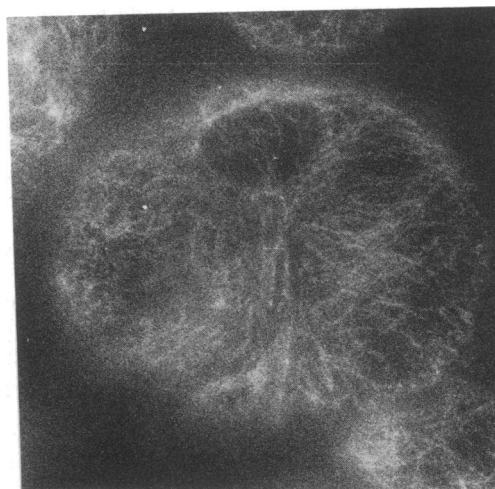


FIG. 1. Microtubule system of the entire exorbital lacrimal acinus from a rat. Immunofluorescence micrograph of an acinus that, after a 220-min incubation in KRB buffer, was fixed, made permeable, and incubated with an anti- α -tubulin as first antibody. The acinar lumen appears as a black area at the upper border of the acinus. ($\times 1000$.)

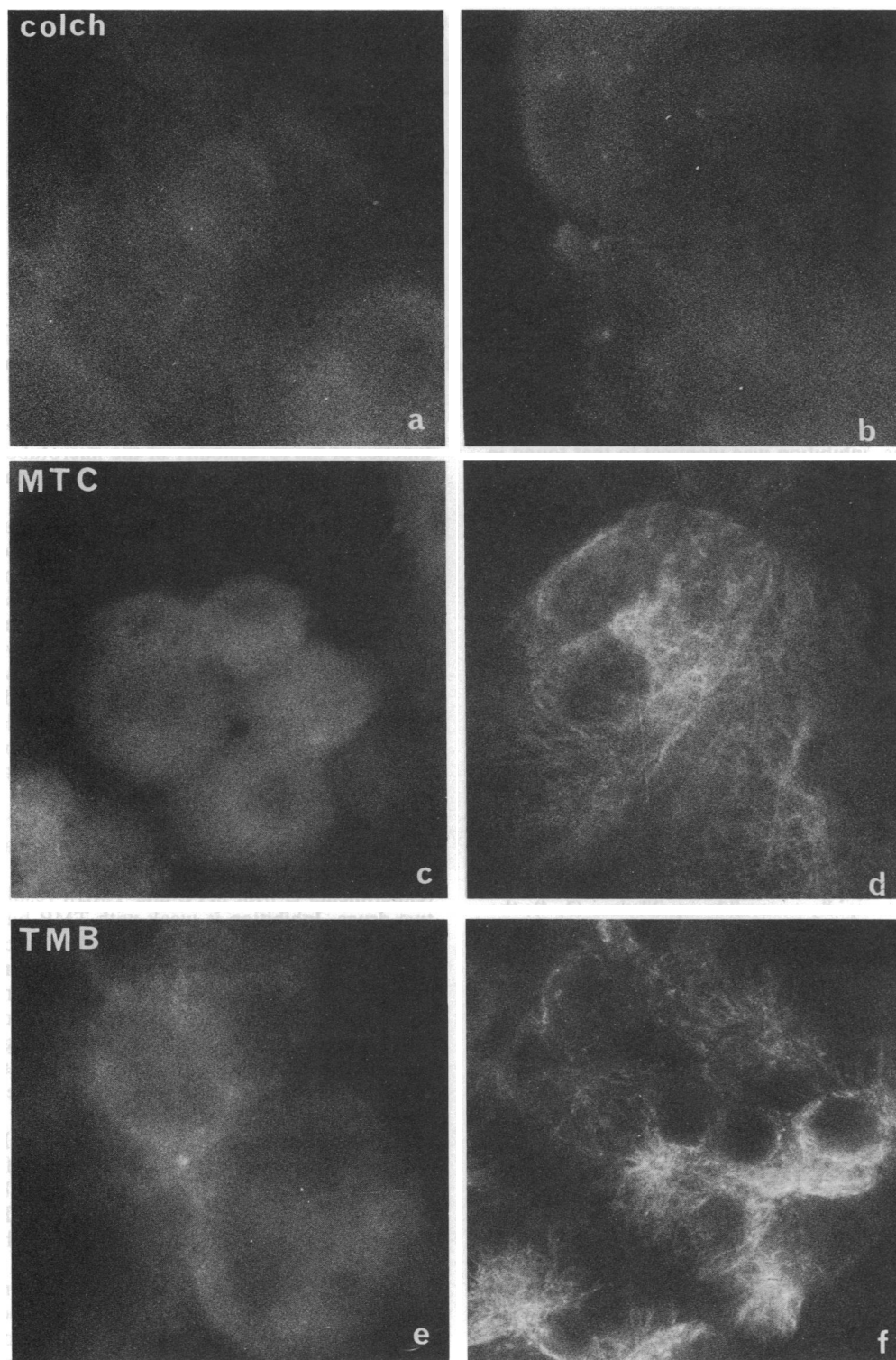


FIG. 2. Effects of colchicine (colch), MTC, and TMB on the microtubule system of exorbital lacrimal acini. Immunofluorescence micrographs of acini (*a*, *c*, and *e*), incubated for 70 min with 1 μ M colchicine (*a*), 25 μ M MTC (*c*), or 100 μ M TMB (*e*) in KRB/BSA buffer. (*b*) Preincubation (70 min) with 1 μ M colchicine, washings, and 145-min incubation in drug-free medium. (*d*) Preincubation (70 min) with 25 μ M MTC, washings, and 40-min incubation in drug-free medium. (*f*) Preincubation (70 min) with 100 μ M TMB, washings, and 25-min incubation in drug-free medium.

Experiment B: Presence of colchicine during the 70 min of prepulse incubation caused an $\approx 80\%$ inhibition of the discharge of labeled proteins. Under similar conditions neither MTC nor TMB displayed any inhibition.

Experiment C: When the drugs were present during the pulse and the first chase period—i.e., maturation and beginning of apical storage—the degree of secretory inhibition varied with the nature of the drug. With colchicine and MTC

inhibition was as strong as in experiment A; with TMB inhibition was weaker.

Experiment D: Presence of the drugs during the second chase period—i.e., end of maturation and apical storage of the secretory granules and that of their discharge—caused only weak inhibition of secretion for lobules incubated with colchicine or MTC and no inhibition with TMB.

Experiment E: When the drugs were present during the

Table 1. Effects of colchicine, MTC, and TMB on the release of newly labeled proteins under 2 μ M carbachol stimulation in rat lacrimal exorbital lobule

Exp.	Presence (+) or absence (-) of drug					Inhibition of net labeled protein secretion, %		
	Prepulse (70 min)	Pulse (10 min)	Chase 1 (60 min)	Chase 2 (60 min)	Carbachol stimulation (60 min)	Colchicine (1 μ M)	MTC (25 μ M)	TMB (100 μ M)
A	+	+	+	+	+	69 (3)	75 (3)	74 (4)
B	+	-	-	-	-	80 (2)	0 (2)	3 (2)
C	-	+	+	-	-	64 (3)	70 (3)	20 (3)
D	-	-	-	+	+	18 (2)	12 (2)	0 (2)
E	-	-	-	+	-	18 (2)	12 (2)	0 (2)
F	-	-	-	-	+	0 (2)	0 (2)	0 (2)

Each value is the mean of two or three separate experiments; number of experiments is shown in parentheses. Control of unstimulated secretion was 4.2–8.7%; control of stimulated secretion was 39–52%. Net secretion equals stimulated secretion minus unstimulated secretion.

final period of maturation and the apical storage of the secretory granules, inhibition was identical to that found in experiment D for each drug.

Experiment F: When the drugs were present only during the protein-discharge period, no observable inhibition occurred with any of the drugs.

Effects similar to those found with TMB have been obtained also with 2,3,4-trimethoxy-4'-carbomethoxy-1,1'-biphenyl (TCB) (10), which is an analogue of TMB with the 4'-OCH₃ radical replaced by—COOCH₃ (§, 4). Furthermore, lumicolchicines β and γ were found to have no effect either on the microtubule network or on protein secretion.

DISCUSSION

Results of the present immunofluorescence study show clearly that the action of these drugs on differentiated secretory cells is exercised at the level of tubulin. All three drugs are known to bind to tubulin—the binding of colchicine being essentially irreversible (1), whereas that of MTC and TMB is characterized by a rapidly reversible equilibrium (3, 4); the reversibility of TMB binding, however, is faster than that of MTC. Furthermore, all three drugs inhibit the formation of microtubules from pure tubulin—colchicine inhibits irreversibly, but MTC and TMB inhibit reversibly. The difference in the drug concentrations necessary for *in vitro* inhibition of microtubule formation is reflected directly in the drug concentrations needed to (i) destroy the microtubular network in lacrimal acini and (ii) exert an inhibitory action on protein secretion in lacrimal lobules. This result strongly suggests that these drugs have destroyed the cellular microtubular system by a mechanism similar to that of their *in vitro* inhibition of microtubule formation from pure tubulin—namely, their binding to tubulin.

The described results demonstrate a relationship between the integrity of the microtubular network and the secretory process within the functional lacrimal acinar cells. The many studies described earlier on the effect of antimicrotubular drugs on cytoskeleton organization have involved either *in vitro* probing of their effect on microtubule formation from purified tubulin (3, 9) or immunofluorescence examination of cultured cells (11–13, see also ref. 14). In this study, we have examined the effects of these drugs in parallel on a biological function, that is, on the secretory pathway, and on cytoskeleton organization. Secretion was measured in lobules of exorbital rat lacrimal glands, whereas immunofluorescence of the microtubular network was studied on cells associated into acini—i.e., cells that had conserved their polarity. Thus, we report the visualization by immunofluorescence of polymerized and depolymerized states of the microtubule network modulated by drugs within exocrine secretory acini. The correlation between the drug effects on secretion and on the microtubular network is striking.

A comparison of the results on protein secretion and on the kinetics of the reversibility of the microtubular system seen by immunofluorescence allows the following three conclusions.

(i) Depolymerization of microtubules during the entire course of the secretory process (labeled protein synthesis, secretory granule maturation, and apical storage) induces a strong 70% inhibition of labeled-protein discharge whether this depolymerization is caused by continuous drug presence (experiment A) or by formation of the irreversible tubulin-colchicine complex (experiments B and C with colchicine).

(ii) Conversely, when the drugs are introduced only after the maturation period of the secretory granules (experiments D and E) or only during stimulation (experiment F) protein discharge is affected only slightly or not at all, even though the microtubular system is depolymerized.

(iii) When TMB or MTC (which bind reversibly to tubulin) are present during the pulse and the initial phase of secretory-granular maturation but are absent during the incubation (experiments C with MTC and TMB), results differ for the two drugs. Inhibition is weak with TMB but is strong with MTC. Meanwhile, the immunofluorescence results have shown that the microtubular system is restored 25 min after removing TMB from the incubation medium. Under these circumstances the microtubular system could have reassembled during the end of maturation and the beginning of apical storage; the observed inhibition is, thus, only partial. For MTC (experiment C), immunofluorescence study showed that the microtubular system appears only \approx 40 min after MTC removal from the incubation medium. In this case, the second maturation period and beginning of apical storage proceed almost totally without any microtubules, and inhibition of protein secretion remains strong. Thus, the maturation phase appears linked to the state of the microtubular system.

In earlier papers (5, 8, 15) in which colchicine (which forms the irreversible complex with tubulin) was used it was shown that (i) destruction of microtubules slowed down the passage of proteins in the ribosomal endoplasmic reticulum–Golgi area; and (ii) secretion granules formed in the presence of colchicine were not discharged. We could not, however, establish the stage at which the secretory granules underwent the modification that made them incapable of exocytosis. The results obtained in the present study, by use of the colchicine analogues that inhibit microtubule polymerization reversibly, led us to the conclusion that it is the maturation stage that requires the microtubules. During this stage, secretory granules seem to acquire an additional property [perhaps by means of a protein system; several recent publications have described protein–organelle relations (16–21)], which allows eventual exocytosis. Without microtubules, granule maturation would be impossible because of granular inability to

acquire this property, rendering these granules incapable of exocytosis.

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